Faculty of Resource Science and Technology

EX VITRO ACCLIMATIZATION OF TISSUE CULTURE BORNEO'S ENDEMIC ORCHID, *VANDA DEAREI*

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Ex vitro acclimatization of tissue cultured Borneo's endemic orchid, *Vanda dearei*

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A report submitted in fulfilment of the requirement for the Degree of Bachelor Science with Honours

Supervisor: Assoc. Prof. Dr. Ho Wei Seng

Resource Biotechnology

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LIST OF ABBREVATIONS

ABA	Abscisic acid
BAP	6-Benzylaminopurine
DAC	Days after culture
DMRT	Duncan Multiple Range Test
CCC	Chlorocholine chloride
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
NAA	Naphthalene-acetic acid
PMA	Phorbol myristate acetate

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ABSTRACT

Vanda dearei belongs to Orchidaceae family which is innate to the Borneo Island. It is well-known due to large, pale yellow and strong fragrant flowers. Hybridists choose *V. dearei* in breeding of the hybrid flowers due to the yellow breeding lines and spontaneous flowering. Modern tissue culture methods such as *in vitro* germination and micropropagation help to produce vast amount of plant products including *Vanda dearei*. However, acclimatization stage is an obstruction of micropopagation technique due to vast amount of plants die. This study was aimed to establish the suitable treatments for acclimatization of *Vanda dearei*. The plantlets were subjected to different acclimatization approaches. There is a control and two treatments [treatment 1 (charcoal and coconut fibre) and treatment 2 (charcoal and peat moss)]. The results show that there was significant higher in percentage of plant survival rate, plant height, number of roots and leaves in treatment 2 compared to control and treatment 1. Hence, it can be concluded that treatment 2 (charcoal and peat moss) is the suitable treatment to acclimatize *Vanda dearei*.

Key words: *Vanda dearei,* acclimatization, plantlets, survival rate

ABSTRAK

Vanda dearei tergolong dalam keluarga Orchidaceae yang semula jadi di Pulau Borneo. Ia terkenal kerana besar, kuning pucat dan kuat bunga wangi. Hybridists memilih *V. dearei* dalam pembiakan bunga hibrid kerana garis pembiakan kuning dan berbunga spontan. Moden kaedah kultur tisu seperti *in vitro* percambahan dan mikropropagasi bantuan untuk menghasilkan jumlah luas produk-produk tumbuhan termasuk *Vanda dearei*. Walau bagaimanapun, peringkat penyesuaian menjadi halangan teknik micropopagation kerana banyak tumbuhan mati. Kajian ini bertujuan untuk menubuhkan rawatan sesuai untuk penyesuaian daripada *Vanda dearei*. Anak pokok tertakluk kepada kaedah yang berbeza. Terdapat kawalan dan dua rawatan [rawatan 1 (arang dan serat kelapa) dan rawatan 2 (arang dan lumut gambut)]. Hasil kajian menunjukkan bahawa terdapat signifikan tinggi dalam peratusan kadar tumbuhan hidup, ketinggian, bilangan akar dan daun dalam rawatan 2 berbanding kawalan dan rawatan 1. Kesimpulannya, rawatan 2 (arang dan lumut gambut) adalah rawatan yang sesuai untuk *Vanda dearei*.

Kata kunci: *Vanda dearei*, penyesuaian, anak pokok, kadar survival

CHAPTER 1

INTRODUCTION

Evolution of orchids in floral morphology, structure and physiological properties begin at late cretaceous allow them to become one of the favourite species for botanists (Tsai *et al.*, 2013). Hence, orchids have the greatest amount of species with approximately more than 25,000 species where they exhibit in abundant diversity of epiphytic and terrestrial growth forms (Hsiao *et al.*, 2011). According to Bhattacharjee and Islam (2014), orchids had become one of the top ranked among all flowering plants due to the attractiveness of the plants and longer life-span compare to other flowers especially *Vanda dearei*.

V. dearei (Orchidaceae) is a tropical epiphytic and monopodial orchid and innate to the Borneo Island (Chan *et al.*, 1994). The huge beautiful petals and strong fragrant flowers produced by the *V. dearei* all over the year cause them rated among the favoured orchid species (Jualang *et al.*, 2014) especially citizens of Borneo as well as some of the Asia countries (Jawan *et al.*, 2010). However, the number of *V. dearei* has been decreased from year to year due to human activities which include mining, urban development, land clearing for agriculture on their natural habitat and illegal collection (Fay, 1994). Therefore, it is crucial to sustain *V. dearei* by modern tissue culture method to increase the number of *V.dearei* because traditional method of orchids propagation are waste of time and tedious process even though huge quantity of seeds were produced but only a few of them germinate under natural situation (Rao, 1977).

Modern plant tissue culture methods include micropropagation can produce large number of plants under controlled environment conditions (Díaz *et al.*, 2010). However, most of the orchids can micropropagate without any problems but show poor acclimatization during *ex vitro* due to transfer of plants from an ideal situation of *in vitro* to *ex vitro* condition (Debergh & Zimmerman, 1991). According to Preece and Sutter (1991), acclimatization is a condition that allow the plant to become autotrophic and growing in an environment with decreased humidity, high light intensity and septic potting mixtures. During acclimatization, high number of plants can die and thus quality plantlets must be acquired under *in vitro* conditions to ensure a high survival rate and suitable growth under *ex vitro* conditions.

However, suitable growing environments such as gradually decreasing humidity and increasing light intensity for *ex vitro* can speed up the propagation process and enrich the quality of the plantlets. According to Franco *et al.* (2007), selection of the potting media is also an important factor to guarantee a high survival rate of the plantlets as potting media provides mechanical support, increase water absorption by the roots (Tortosa, 1990). Hence, it is significant to select suitable potting media with great aeration, low septicity and accurate acidity (Díaz *et al.*, 2010).

The significance in carrying out *ex vitro* acclimatization of tissue cultured Borneo's endemic orchid, *V. dearei* is to optimize the potting mixtures. Furthermore, successfully cultured *V. dearei* in suitable potting mixtures can imply in further study to use as a reference.

The objectives of this study are:

i.) To investigate the effect of different potting mixtures on *ex vitro* acclimatization of *Vanda dearei.*

ii.) To determine the suitable potting mixtures with high survival rate on *ex vitro* acclimatization of *Vanda dearei.*

CHAPTER 2

LITERATURE REVIEW

2.1 Orchidaceae

Evolution of orchids in floral morphology, structure and physiological properties begin at late cretaceous allow them to become one of the favourite species for botanists (Tsai *et al.*, 2013). Hence, Orchidaceae have the greatest amount of species with approximately more than 25,000 species where they exhibit in abundant diversity of epiphytic and terrestrial growth forms (Hsiao *et al.*, 2011). Epiphytic orchids have aerial roots to hold on the substrate for water and mineral absorption whereas terrestrial orchids take in nutrients directly from the soil (Black, 1973). Orchidaceae are subdivided into five subfamilies which include Apostasioideae, Cypripedioideae, Epidendroideae, Spiranthoideae, and Orchidoideae (Dressler, 1993). There are two groups of orchids which include sympodial and monopodial. Sympodial orchids are those grow from a stem which is horizontal such as *Cattleya*, *Cymbidium*, *Dendrobium* and *Oncidium* whereas monopodial orchids are those grow vertically with a single upright stem with one leaf following another on opposite sides of the centre such as *Phalaenopsis* and *Vanda* (Jang *et al.*, 2015). Figure 2.1 shows the different growth form of orchids.

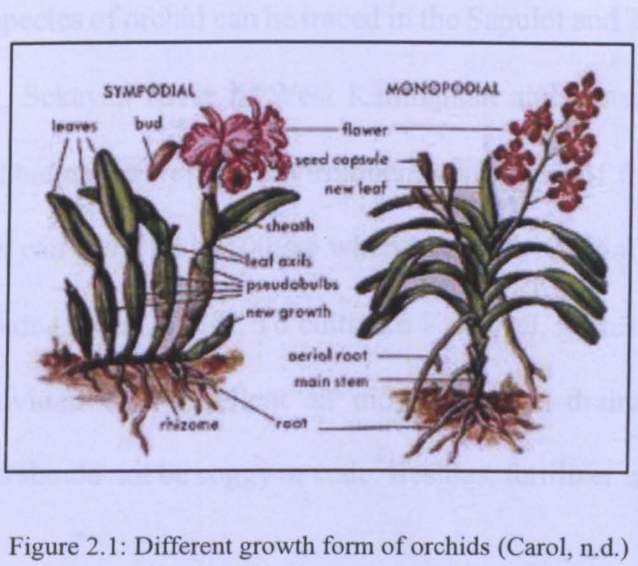

Figure 2.1: Different growth form of orchids (Carol, n.d.)

According to Bhattacharjee and Islam (2014), Orchidaceae had become popular among botanists and researchers due to their attractiveness such as variation in flower species, medicinal properties and longer life span. Hence, there are illegal collection of orchid species all around the world that lead to decrease number in orchid species (Coates *et al.,* 2007). Thus, the discovery of modern tissue culture methods such as *in vitro* germination and micropropagation are crucial to increase the number of orchid species.

Traditional techniques of propagation of orchids are slow and laborious. Orchids are able to produce thousands of seeds but it is very hard to grow orchid from the seed because there is only small amount of nutrients contain inside the seed (Arditti & Ghani, 2000). However, there was a major breakthrough happened in 1917 when Dr. Lewis Knudson of Cornell University found that the seedlings could be cultivated by growing in sugar-based solid media (Arditti & Ghani, 2000). Thus, efficiency of the micropropagation systems using tissue culture techniques has been increased and this method has been widely used in the propagation of Orchidaceae (Arditti & Ghani, 2000).

2.1.1 *Vanda dearei*

Vanda dearei (Orchidaceae) is a tropical epiphytic and monopodial orchid and innate to the Borneo Island. This species of orchid can be traced in the Sapulot and Tomani area in Sabah, Kuching in Sarawak, Sekayan River in West Kalimantan and Kutai in East Kalimantan (Chan *et al.*, 1994). Due to the tropical environment conditions of the Borneo Island, the stems of the *V. dearei* can grow up to 100 cm whereas the leaves can grow up to 35-45 cm long by 3.0-3.8 cm wide (Motes, 1988). To cultivate *V. dearei*, Motes (1988) stated that *V. dearei* should be provided with excellent air movement and drainage system, and the conditions of the roots should not be soggy or stale. Besides, fertilizer can be applied weekly when the plants are actively growing when there are new roots and leaves (Motes, 1988). Figure 2.2 shows the flower of *Vanda dearei*.

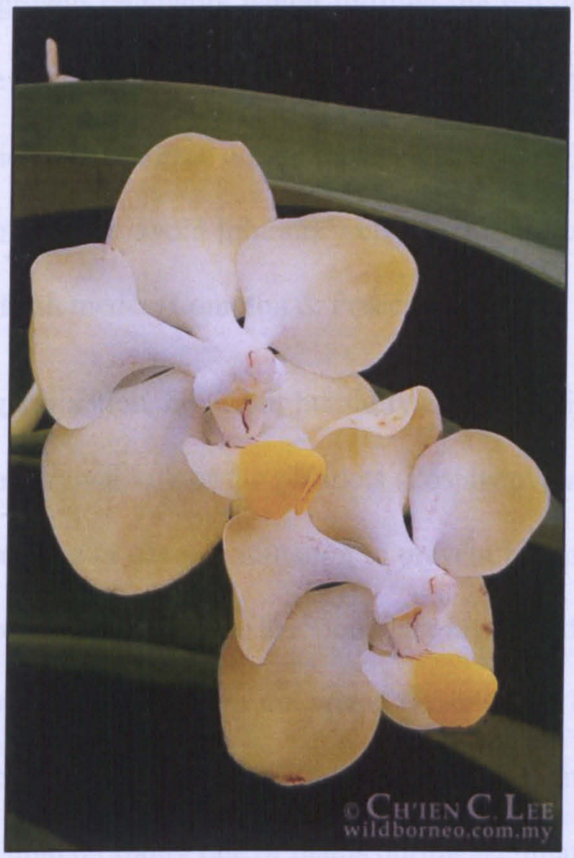

Figure 2.2: *Vanda dearei* (Lee, 2017)

2.2 Propagation of orchid

2.2.1 *In vitro* germination

Traditional method of vegetative propagation of orchids is time consuming and tedious process. Although orchids produced huge quantity of seeds, only few of them germinate under natural situation due to insufficient nutrients and require mycorrhizal association (Rao, 1977). Hence, modern tissue culture methods such as *in vitro* germination and micropropagation were used to increase the number of orchid species. Tissue culture is a technique use to culture a plant from explant of the plant organs such as meristems, leaves, flower, flower stalks or buds (Buenavista *et al.*, 2010). According to Gamborg *et al.* (1976), the success of tissue culture depends on the explant origin and culture medium. The most widely used culture medium was Murashige-Skoog (MS) because MS contains mineral salts, sucrose, vitamins and growth regulators which fulfilled the physiological needs of many plant cells in the culture (Gamborg *et al.*, 1976). Phenolics compound that released from the orchids can become toxic when oxidized during tissue culture (Chugh *et al.*, 2009). However, there is an alternative method to avoid inhibitory effects of the toxic compound by quick transfer the explants to fresh media (Compton & Preece, 1986).

During *in vitro* germination, higher air humidity and lower light concentration in the culture will increase the survival rate of the plantlets (Pospisilova *et al.*, 1999). Moreover, closed vessel was used during *in vitro* germination to prevent microbial contamination and lower air turbulence in order to enhance the leaf boundary layers and control carbon dioxide and oxygen from flowing in and out from the vessel (Pospisilova *et al.*, 1999). The ideal conditions of *in vitro* germination ensure high survival rate of plantlets can be obtained in order to compensate the high mortality rate of plants during acclimatization.

2.2.2 Acclimatization of orchid

Most of the orchids can micropropagate *in vitro* without any problem but show poor acclimatization during *ex vitro* (Debergh & Zimmerman, 1991) due to transfer of the plants from an ideal situation of *in vitro* to *ex vitro* condition. They need some time to adapt new environment in order for them to survive. According to Preece and Sutter (1991), acclimatization is a condition that allow the plant to become autotrophic and growing in an environment of decreased humidity, high light intensity and septic potting mixtures. During acclimatization, high number of plants die and thus quality plantlets must be obtained under *in vitro* conditions to ensure a high survival rate and suitable growth under *ex vitro* conditions.

Furthermore, there are different acclimatization methods have been carried out such as using different *in vitro* culture media, substratum, concentration of agar and sucrose in the medium (Ortega-Loeza *et al.*, 2011). According to Franco *et al.* (2007), selection of the potting media is an important factor to ensure high survival rate of the regenerated plantlets as potting media provides mechanical support, increase water absorption by the roots (Tortosa, 1990). Therefore, different potting media has been studied to enhance the survival rate of orchids during acclimatization.

2.3 Factors affecting *ex vitro* tissue cultured of orchid

2.3.1 Sugar alcohol in the medium

Plant cell, tissue and organ culture require sucrose to supply carbon source as sucrose ensures bigger leaves can be produced in order to generate larger storage compounds (Hazarika, 2003). However, excessive sucrose appeared to be stressful for the shoots, where the plants showed poor formation of leaves (Hazarika, 2003).

Studies showed that addition of alcoholic sugar such as sorbitol, mannitol, maltitol, erythritol, and hydrogenated starch hydrolysates on *Dendrobium spectabile* which is an epiphytic orchid has the maximum quantity of leaf produced (Santoso *et al.,* 2014). Furthermore, Santoso *et al.* (2014) also stated that leaf colour of orchid appeared to be green and bigger when the orchid was subjected to alcoholic sugar and antimicrobial supplementation during acclimatization. Santoso *et al.* (2014) also reported that the orchid seed will die if there is absent of alcoholic sugar and antimicrobial supplementation due to microbial contamination and water stress.

2.3.2 Growth regulators

Plant growth regulators, auxin such as indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) will stimulate shortening of the internode, leaf size reduction, strengthening of green colouration of leaves and thickening of roots.

According to Smith *et al.* (1991), he stated that growth regulators can be applied in micropropagation to prevent wilting. For instance, Smith *et al.* (1991) proposed that paclobutrazol (0.5-4 mg) in the rooting medium will reduce stomata apertures, enhanced epicuticular wax, increased roots and stems development, lower the risk of wilting after relocated to potting mixtures, and enhanced chlorophyll concentration of leaf.

Studies showed high concentration of auxins and low concentration of cytokinins can induce root formation in epiphytic orchid (Sharma & Vij, 1997). For instance, active biosynthesis of IAA in the roots of epiphytic orchid had been proposed for *Vanda* and *Aranda* species (Zhang *et al.*, 1995). Novak *et al.* (2014) stated that interaction of endophytes with auxin play an important role in symbiotic relationship of orchid whereby orchid plant strictly depends on those bacteria and fungus to allow seed germination and root development to occur. Furthermore, Das *et al.* (1990) described that direct rooting of tea shoot was accomplished by dipping the cut end in IBA (50 mg 1-1, 30 minutes) and then planting the tea shoot in a soil: peat moss::1:1 mixture. By this way, the survival rate of tea shoots can be enhanced compare to those rooted under *in vitro* conditions.

2.3.3 Antitranspirants

Antitranspirants such as abscisic acid (ABA) can decrease water loss during acclimatization (Hazarika, 2003). Moreover, abscisic acid can enhance formation of new leaves on acclimatized plants once the plant transfer from *in vitro* to *ex vitro* condition (Hazarika, 2003). Agents that cover leaf surface which include glycerol, paraffin and grease improve *ex vitro* survival of some orchid species (Selvapandiyan *et al.,* 1988).

Other than abscisic acid (ABA), antitranspirants such as phenylmercuric acetate (PMA) and chlorocholine chloride (CCC) can reduce water loss as well as encouraged stomatal closure and delayed wilting in tomato plants (Mishra & Pradhan, 1972).

2.3.4 Potting media

There are various kind of potting medias used for epiphytic and terrestrial orchids in the pots (Chandra *et al.*, 2010). According to Franco *et al.* (2007), selection of the potting media is an important factor to ensure a high survival rate of the regenerated plantlets as good potting media provides mechanical support and air absorption by the roots (Tortosa, 1990). For instance, Beardsell *et al.* (1979) stated that it is important to consider the water-holding capacity of the potting media as most of the pots have limited volume of soil to store water. However, waterlogging can happen in the lower part of the pots if the potting media has bad porosity (Beardsell *et al.*, 1979). Hence, it is also important to select suitable potting media with great porosity as well as great water-holding capacity (Díaz *et al.*, 2010). Other than that, selection of potting media must be resistance to decomposition and good aeration (Franco *et al.,* 2010).

However, there are different type of potting medias used to acclimatize such as soil, substrate, cultivated soil and compost. Therefore, it is important to select suitable potting media according to the different needs of the plants. Table 2.1 shows the evolution in growing media in time. Substrate is a solid and porous, natural or artificial material, mixed or without which allow plant growth under maintained environment situations (Abad, 1989). Example of substrates include rock wool, styrofoam, charcoal, peat moss, sphagnum moss and coconut fibre.

From Table 2.1, number of pores and humus of substrate is higher compare to soil. High number of pores indicated good drainage system while high number of humus indicated good nutrients content (Beardsell *et al.*, 1979). Hence, substrate has better drainage system and nutrients content compare to soil. Furthermore, substrate does not require to control the weed grow and infection compare to soil which in turn reduces the risk of plants expose to diseases. Next, pH and nutrient can be controlled by substrate compare to soil. Lastly, plants grow in substrate has better growth and health compare to soil. Therefore, substrate is a suitable growing media to acclimatize *Vanda dearei*. *V. dearei* is an epiphytic orchid, it depends on aerial roots to hold on the potting media for water and mineral absorption. Thus, substrates such as coconut fibre, charcoal or peat moss are suitable potting media to acclimatize *V. dearei*. Table 2.2 shows the pros and cons of potting medias and Table 2.3 shows the studies of potting mixtures used in various orchid species.

Table 2.1: Evolution in growing medias in time (Verdonck, 1981)

	Soil	Substrate
Example	Sand	Peat
Humus & pores	Increasing →	
Weed control	Yes	No
Infection	Great	No
pH & nutrient control	No	Possible
Growth and health	Increasing →	

Table 2.2: Pros and Cons of potting medias

Potting Media	Pros	Cons	Author
Charcoal	• Decompose slowly	• Dusty • Holds little moisture	Frowine, 2005
	• Store nutrients • Good aeration • Good drainage system		Tsamboura kis, 2012
	• Maintained pH levels • Enhanced nitrogen uptake		Eymar *et al.*, 2000
Coconut fibre	• Decompose slowly • Good water retention • Good anchorage capacity	• Drainage system is not good compare to other potting media	Frowine, 2005
Peat moss	• Good water retention • Good aeration	• Fast decomposition • Retain too much water if packed too compact in the pot.	Frowine, 2005

Table 2.3: Studies of potting mixtures used in various orchid species

Potting mixtures	Description	Authors
Mixture of charcoal pieces (5-7 mm), brick chips or mosses at (1:1) ratio	- Suitable for Epiphytic orchids - Around 80-85 % survival rate after 3 months	Deb & Imchen, 2010
Mixture of moss and decayed wood or forest litter along with charcoal pieces and brick chip.	- Suitable for Terrestrial orchids - Around 80-85 % survival rate after 3 months	Deb & Imchen, 2010
Mixture of coconut fibre and charcoal in (1:1) ratio	About 90 % survival rate for *Aranda* Wan Chark Kuan "Blue" x *Vanda coerulea* Grifft. ex. Lindl. after 4 months	Gantait & Sinniah, 2012
Charcoal	Stimulate rooting and prevent the toxic substances from damage the orchids by adsorb the toxic substances • *Vanda coerulea* • *Vanilla planifolia* • *Cymbidium sinense*	 • Seeni & Latha, 2000 • George & Ravishankar, 1997 • Chang & Chang, 2000
Coconut fibre	76 % of survival rate for *Cattleya trianae*	Franco *et al.*, 2007

CHAPTER 3

MATERIALS AND METHODS

3.1 Source and preparation of plant material

Plantlets were obtained from the Forest Genomics and Informatics Laboratory in University Malaysia Sarawak that have accomplished *in vitro* propagation process. Selection of plantlets were done to choose healthy plantlets with roots. Next, the plantlets were standardised in Murashige-Skoog, (MS) media (Murashige & Skoog, 1962) with 1.0 g/L 6-Benzylaminopurine (BAP) for a week before transfer to the pots.

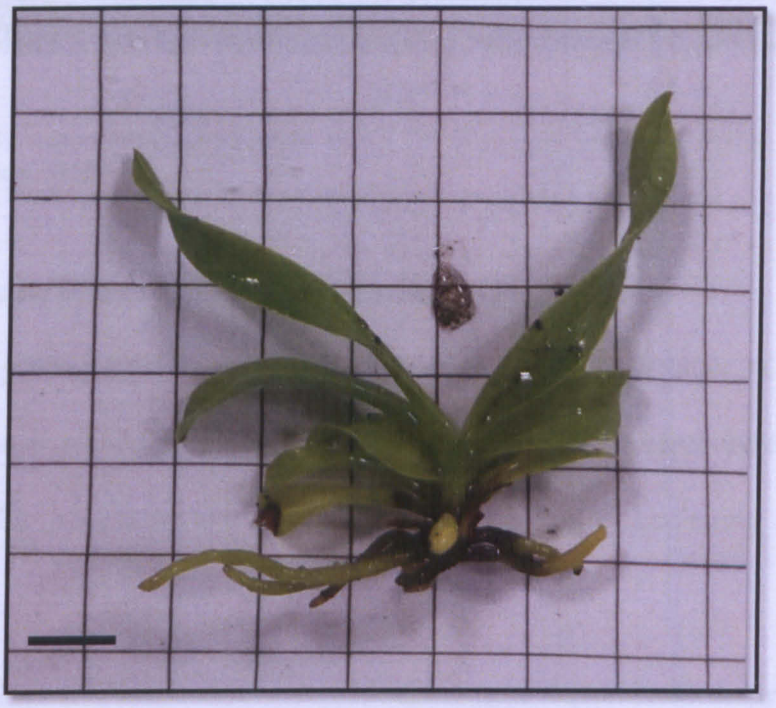

Figure 3.1: Source of plant materials from FGiL; Scale bar: 1 cm